Carbon-source-dependent synthesis and composition of biosurfactant synthesized by *Pseudozyma antarctica**

Włodzimierz Bednarski, Marta Narwojsz, Marek Adamczak, Radosław Nawotka

Department of Food Biotechnology, University of Warmia and Mazury in Olsztyn,
ul. J. Heweliusza 1, 10-718 Olsztyn, Poland
Corresponding author: Włodzimierz Bednarski, telephone: (+48 89) 523 3687, fax (+48 89) 523 3838,
e-mail wlodzimierz.bednarski@uwm.edu.pl

ABSTRACT

Pseudozyma antarctica (formerly *Candida antarctica*) ATCC 28323 was cultivated in preselected conditions in a medium containing glucose and products of enzymatically hydrolyzed lactose in milk permeate (after ultrafiltration) supplemented with the waste fats (fish, pork and post-refining fatty acids) to induce and intensify biosurfactant synthesis. The yield (12.0–26.7 g/dm^3) of biosurfactant synthesis and the fatty acid composition of its hydrophobic fractions depended on carbon sources, i.e. waste fats listed above which have been added to the medium. The obtained results indicate that the fatty acids from the waste fats used seemed to be directly incorporated in the mannosylerithritol lipids. The main fatty acids present in biosurfactant were C16:0, C18:0, C18:1, C18:2.

INTRODUCTION

In the bioremediation of hydrocarbon-contaminated soil or water, chemical agents are increasingly and more frequently replaced by biological substances, e.g. biosurfactant synthesized by some bacterial or yeast species (Benincasa et al. 2002; Deleu and Paquot 2004). Biosurfactants are also utilized in the cosmetic and the food industries, mainly as emulsifiers and anti-microbial preparations. They also possess antimicrobial properties and change the adhesion of microorganisms.

The yield of microbial biosurfactant depends on microorganism properties, medium composition and culture conditions (Adamczak and Bednarski 2000; Cavalero and Cooper 2003). While designing a medium composition, an important biotechnological challenge is the selection of carbon source (hydrophilic – mainly saccharides and hydrophobic – *n*-alkanes, lipids). Possible medium components are also analyzed for their availability and price. In order to decrease the costs of medium preparation, food industry byproducts (whey and fat, e.g. soapstock, post-refining fatty acids) are utilized (Bednarski et al. 2004; Benincasa et al. 2002).

Chemical composition of the medium determines the synthesized biosurfactant structure and properties (Adamczak and Bednarski 2000; Morikowa et al. 2000; Cavalero and Cooper 2003). According to Cavalero and Cooper (2003), the composition of the hydrophobic fraction of sophorolipid synthesized by *Candida bombicola* depends on the type of fatty acids present in the medium – mainly long-chain fatty acids (C16 and C18) which are directly included into the biosurfactant structure.

The aim of the study was to determine the effect of the type of the applied lipids and the composition of their fatty acids on the productivity of the biosurfactant synthesis and on the fatty acid composition in the lipid fraction of the glycolipid synthesized by *Pseudozyma antarctica*.

MATERIAL AND METHODS

Carbon source used in the experiments

The following products from Polish factories were used in the experiments as the carbon source: A. hydrophilic: post-ultrafiltration dried milk permeate, Dairy Plant in Wolsztyn; B. hydrophobic: post-refining fatty acids, Fat Plant in Lejkowo; waste pork fat (a pâté production byproduct), meat processing plant in Olsztyn; waste fish oil, Fish Processing Plant, Big Fish S.A. in Gniewno.

Preparation of a post-ultrafiltration milk permeate solution

A calculated dose of permeate was dissolved in water to obtain a 8% (w/v) lactose solution. After adjusting the acidity to

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pH 5.5 (1 M HCl solution), the permeate was de-proteinated at 95°C and the obtained suspension was filtered through cotton wool. Lactose hydrolysis in obtained filtrate was carried out at 5°C for 24 h with a β-galactosidase preparation – Maxilact 1000 (DSM Food Specialties, Holland).

After lactose hydrolysis, the obtained solution was supplemented with (w/v): waste fat – 10%, $NaNO_3$ – 0.2%, yeast extract – 0.1%, KH_2PO_4 – 0.02%, $MgSO_4 \times 7H_2O$ – 0.02%. The initial acidity of the medium equaled 4.8 – 5.5 and was not adjusted. The obtained medium was distributed at a volume of 100 cm^3 into 500 cm^3 Erlenmeyer flasks and sterilized at 121°C for 15 min.

Preparation of yeast inoculum

Pseudozyma antarctica ATCC 28323 was cultured on wort slants (Fluka) at 30°C for 96 hours. Yeast biomass was washed out from the slants with a 10 cm^3 saline solution, transferred into a 100 cm^3 of the medium without lipids addition and cultured with shaking (200 rpm) at 30°C in an orbital shaker (type G-25, New Brunswick).

Yeast cultivation

The yeast inoculum (10% v/v) was added to the production medium and cultivation was performed by the submerged method with shaking for 144 hours under conditions mentioned above.

During the cultivation, every 24 hours the samples (about 30 cm^3) were taken to determine the yeast growth (dry matter determination after drying at 105°C), the saccharide content (Bertrand method), and surface tension with a stalagmometer (Adamczak and Bednarski 2000).

Separation and purification of biosurfactant (glycolipid)

The post-culture medium was separated by centrifugation (3000×g/10 min 10°C). Biosurfactants were separated from the post-culture medium with ethyl acetate added in the 1:2 (v:v) amount (Kitamoto et al. 1990 a, b).

The raw glycolipid fraction was separated using thin-layer chromatography on a silica gel plate G-60 (Merck) in the developing system containing a chloroform:methanol:water mixture (65:12:2, v:v:v). Chromatograms were stained in the iodine atmosphere and glycolipids were identified with the anthrone reagent (2% (w/v) anthrone in concentrated sulfuric acid) and by heating at 115°C for visualization (blue spots). The spots of the glycolipids were scratched from the plates and then the lipids were eluted from the gel with 40 cm^3 of chloroform.

Analysis of the fatty acid composition in the waste fat and biosurfactant

The methyl esters of the fatty acids were separated by gas chromatography (HP 6890 N with FID; Supelcowax 10, 30 m × 0.32 mm; detector temperature of 250°C; injector temperature of 225°C; column temperature of 180°C; carrier gas – helium). The methyl esters of the fatty acids were prepared with the Peisker method modified by Żegarska et al. (1991).

Statistical analysis

The results of the experiments are the mean value of at least three replicates. Standard deviations did not exceed 5% of the recorded values. Analysis of variance was applied to determine differences between the mean values of the data. Significance was determined at 99% probability (Snedecor and Cochran 1980).

RESULTS AND DISCUSSION

While searching for possible ways of improving the productivity and decreasing the cost of biosurfactant synthesis by microorganisms, it was determined that a medium should be composed of inexpensive hydrophilic carbon sources. Considering the above, the food industry byproducts such as post-ultrafiltration milk permeate and post-refining fatty acids, waste animal fat obtained from the production of pork pâté and fish oil were used in the study.

The glycolipids were not synthesized in the medium supplemented only with lactose (data not published) due to the fact that there are difficulties in assimilation of lactose by *P. antarctica*. To enable the growth of the yeast, this saccharide was hydrolyzed by β-galactosidase. Biosurfactants synthesized by *P. antartica* were described as mannosylerythritol lipids (MEL) and characterized for the first time by Kitamoto et. al. (1990a, b).

The production of MEL is not growth-associated and usually yeast first grows on the saccharides, and begins to produce the glycolipid when it reaches the stationary phase. Various aliphatic hydrocarbons and vegetable oils can be used for synthesis of MEL (Kitamoto et al. 1990b, 1999). Based on the results of the study, the medium composition (including the type of used hydrophobic carbon source) determines the productivity of biosurfactant synthesis as well as yeast biomass growth and sugar consumption (Table 1). The most favorable yield of biosurfactant (26.7 g/l) was obtained in the yeast culture in a medium supplemented with post-refining fatty acids (Table 1).

Considerable differences in the productivity of biosurfactant are not explicitly confirmed by the capacity of biosurfactants to decrease the surface tension of the post-culture liquid. This suggests that not only the volume of the synthesized biosurfactant, but also its chemical composition, is likely to determine their predisposition to decrease the surface tension. Obtained results of surface tension reduction *per se* are not correlated to the yield of glycolipid synthesis and there is no difference in surface tension reduction between the used media (Table 1). The correlation between the surface tension decrease in the post-culture liquid of *P. antarctica* and the medium composition and process conditions was confirmed in a previous paper on this issue (Adamczak and Bednarski 2000).

In line with the aim of the study, the composition of the fatty acids contained in the medium lipids was analyzed and then compared with that of the fatty acids contained

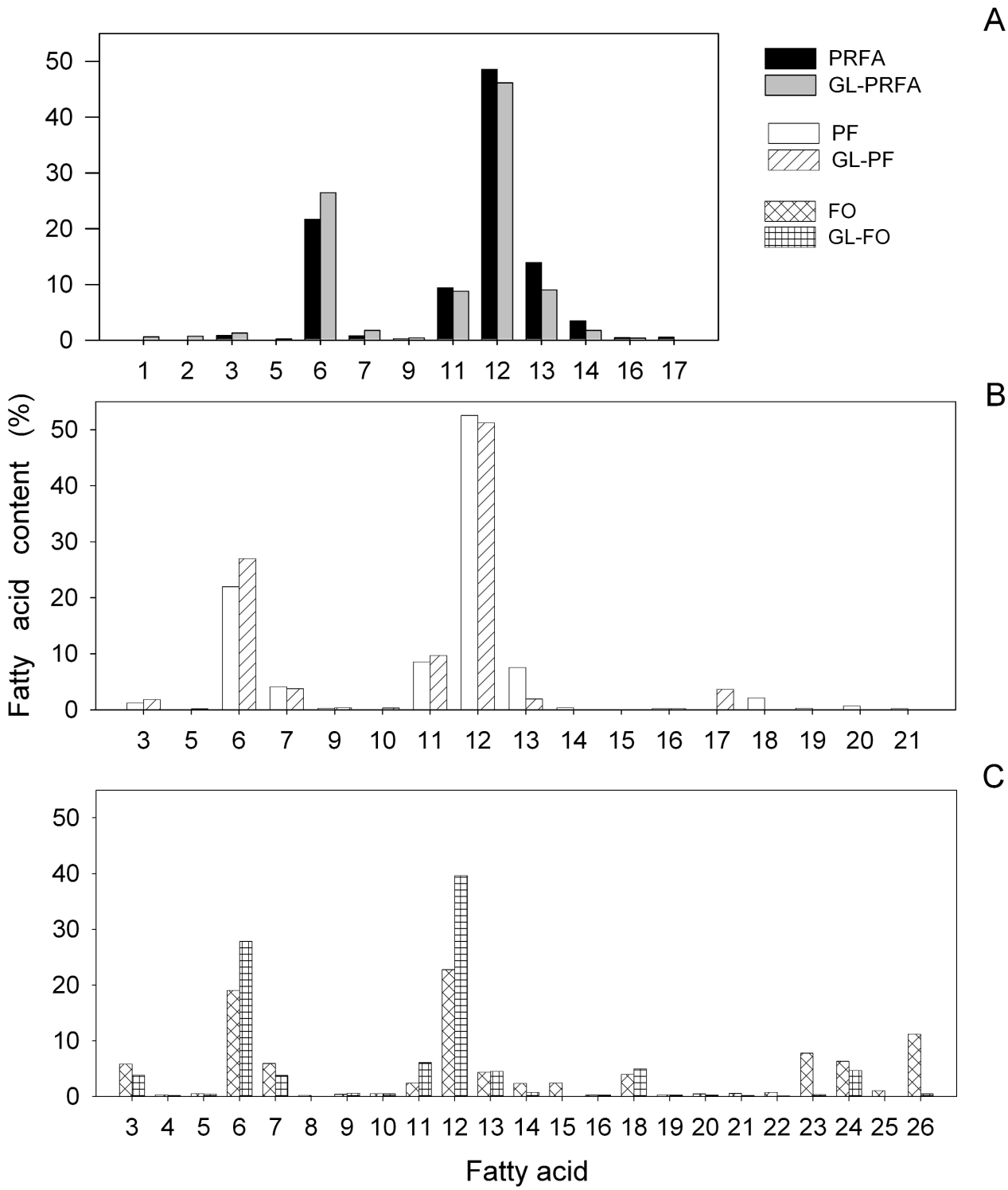

Figure 1. Fatty acid content in the glycolipids (GL) synthesized by *Pseudozyma antarctica* after cultivation in the medium supplemented with waste fats: A. PRFA – post-refining fatty acids; B. PF – pork fat; C. FO – fish oil. Fatty acids: 1- C10, 2-C12, 3-C14, 4-C14:1, 5-C15, 6-C16, 7-C16:1, 8-C16:4, 9-C17, 10-C17:1, 11-C18:0, 12-C18:1, 13-C18:2, 14-C18:3, 15-C18:4, 16-C20:0, 17-C20:1, 18- C20:1 *n*9, 19-C20:1 *n*7, 20-C20:2, 21-C20:4 *n*6, 22-C20:4 *n*3, 23-C20:5, 24-C22:1, 25-C22:5, 26-C22:6. The results are the mean value of at least three replicates. Standard deviations did not exceed 3% of the recorded value.

Table 1. Synthesis of biosurfactant by *Pseudozyma antarctica* after a 144-hour cultivation in the media with a 10% waste fat supplement.

Medium	Yield of biosurfactants synthesis [g/l]	Growth of yeast [g/l]	Surface tension of the initial medium* [mN/m]	Surface tension of post-culture liquid* [mN/m]	Saccharide utilization** [%]
A	26.7	17.7	53.9	31.8	63
B	12.4	14.6	78.9	57.6	54
C	12.0	15.6	80.7	60.2	54

* surface tensions determined using a stalagmometer.

** determined by the Bertrand method.

A – medium with post-refining fatty acids; B – medium with waste pork fat; C – medium with waste fish oil.

The results are the mean values of at least three determinations. Standard deviations did not exceed 5% of the recorded values.

in the lipid fractions of the glycolipid synthesized by *P. antarctica* (Figure 1). It was shown that glycolipid synthesized by *P. antartica* and biosynthetic pathway of fatty acids for MEL were quite different to those generally known in microorganisms: the unique chain shortening pathway participates in MEL biosynthesis (Kitamoto et al. 1998).

Presented data do not confirm this observation and suggest intact incorporation of fatty acids into the glycolipid. The majority of the fatty acids present in the glycolipids were found to be built directly from the medium, but statistically important differences were found in fatty acid composition of the substrate and glycolipids ($p = 0.01$). Glycolipids for the analysis of fatty acid composition were separated from the substrate by thin layer chromatography. Only medium chain fatty acid could be synthesized *de novo*, and only in case of the medium supplemented with post-refining fatty acids. For example, C10 and C12 acids were found in the glycolipid synthesized by *P. antarctica* in the medium supplemented with post-refining fatty acids, but not in the post-refining fatty acids (Figure 1). Based on these results, it was found that the particular fatty acids are directly built into the glycolipid structures to a varied degree. This is likely to be determined by the availability of the fatty acids present in the used lipid substrates (Figure 1). These observations suggest that under the presented experimental conditions the mechanism of lipids incorporation can be different to that described in the literature.

It was observed that the number of glycolipid fractions detected by TLC depends on the hydrophobic substrate added to the medium (Figure 2). Following the chromatographic separation of the glycolipid samples synthesized by *P. antarctica* on the medium with the post-refining fatty acids, the presence of four fractions differing in composition of fatty acids was shown (Table 2). According to the first publication describing mannosylerithritol lipids synthesized by *P. antarctica*, they gave four spots corresponding to MEL-A ($Rf = 0.77$), MEL-B ($Rf = 0.63$), MEL-C ($Rf = 0.58$), MEL-D ($Rf = 0.52$) (Kitamoto et al. 1990b). The pattern of the extracellular lipids

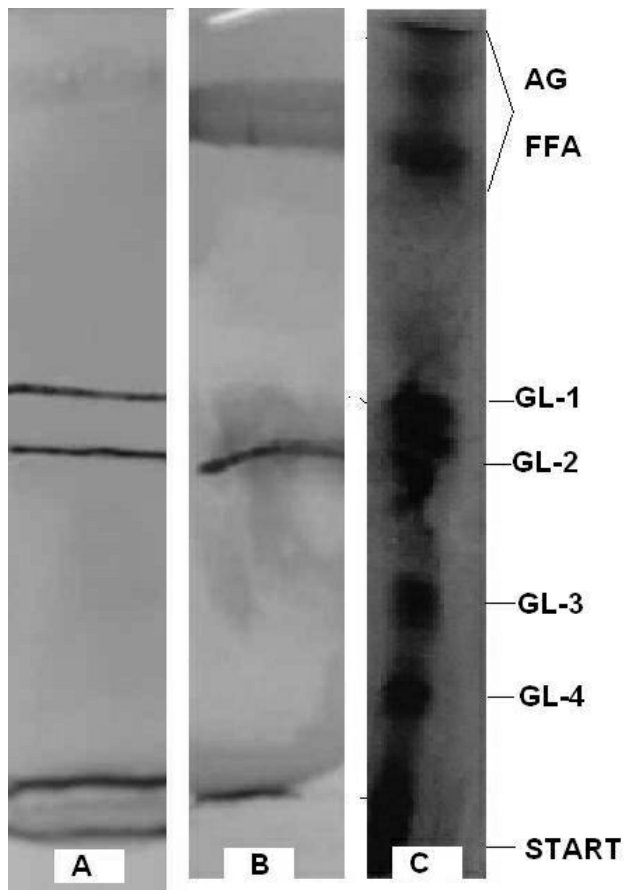

Figure 2. Thin layer chromatography (TLC) of isolated glycolipids from the medium supplemented with waste: A. fish oil, B. pork fat, and C. post-refining fatty acids, detected by using an anthrone reagent. AG-acylglycerols; FFA-free fatty acids; GL-1, 2, 3, 4- glycolipid fractions.

obtained in presented experiments was as follows: GL-1 ($Rf = 0.57$), GL-2 ($Rf = 0.48$), GL-3 ($Rf = 0.31$), GL-4 ($Rf = 0.14$). The Rf values differed also from those reported, for example, by Kawashima et al. (1983), and

Table 2. Fatty acid content in the glycolipid fractions synthesized by *Pseudozyma antarctica* cultured in the medium with a 10% supplement of post-refining fatty acids.

Fatty acids	Fatty acid content (%) in the glycolipid fractions			
	I	II	III	IV
C10	nd	0.69	nd	nd
C12	nd	0.77	nd	0.86
C14	0.39	3.66	1.58	2.68
C15	nd	0.51	0.67	1.27
C16	26.48	63.81	39.95	49.25
C16:1	0.80	0.95	nd	2.11
C17	nd	0.20	nd	nd
C18:0	9.54	6.03	22.50	22.60
C18:1	49.06	17.57	29.34	19.38
C18:2	11.14	5.13	3.11	1.84
C18:3	1.80	0.67	nd	nd
C20:0	0.30	nd	1.14	nd
C20:1	0.49	nd	1.72	nd

The results are the mean values of at least three replicates. Standard deviations did not exceed 3% of the recorded values.
nd – not detected.

from those estimated from the data presented by Rau et al. (2005). The differences were probably caused by the difference in fatty acid composition of glycolipid synthesized by the variety of strains. Another reason could be the differences between the strains used in respective experiments.

These differences can determine the functional properties of the particular glycolipid fractions, e.g. the emulsifying property, foam productivity and stability. The study results are preliminary and require further research. It should be noted, however, that crude preparations of the biosurfactant synthesized by *P. antarctica* exhibit the above-mentioned properties. Foam obtained from a 0.1% biosurfactant solution was very stable (over 30 min). Moreover, the emulsifying properties of the obtained biosurfactants were preliminarily confirmed.

An important problem for further investigation will be MEL downstream processing and separation from the medium. It was found that the yield of MEL separation could be from 4 to 93% (w/v) and the purity could be 100% (Rau et al. 2005). In the presented experiments the yield of separation was less than 1% (w/v).

CONCLUSIONS

Based on the experiments which have involved culturing *P. antarctica* on the medium supplemented with lactose hydrolyzate from the post-ultrafiltration milk permeate, post-refining fatty acids or waste fat i.e. pork fat or fish oil, it was shown that the type and the composition of the fatty acids from the hydrophobic carbon source determine the process productivity and the fatty acid composition of the lipid fraction in the obtained biosurfactants.

Following the chromatographic separation of the biosurfactant samples synthesized by *P. antarctica* on the medium with post-refining fatty acids, it was shown that they are a mixture of four glycolipid fractions which lipid fractions differ in the composition of the fatty acids. The obtained biosurfactants reduced surface tension and exhibited foam forming and emulsifying properties.

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